List of Publications
RESEARCH PUBLICATIONS:


- Ashreeb Naqshbandi, Sana Rizwan and Farah Khan. Dietary supplementation of flaxseed oil ameliorates the effect of cisplatin on rat kidney (communicated) *Nutrition Research*.

- Ashreeb Naqshbandi, Sana Rizwan, Md. Furqan and Farah Khan. Dietary fish oil supplementation ameliorates the effect of cisplatin on brush border membrane enzymes and antioxidant system in rat intestine (communicated) *Act Biochim Pol*.

- Mir Kaisar, Ashreeb Naqshbandi, Mohd Fareed and Riaz Mahmood. Oral administration of potassium bromated, a food additive alters renal redox, metabolic status and induces oxidative stress in rats. (under revision) *Food chemistry*.
PRESENTATIONS:


- Sana Rizwan, Ashreeb Naqshbandi, Md. Wasim Khan, and Farah Khan. Studies on the protective effect of flaxseed oil on Arsenate induced toxicity in rat liver. National symposium on current trends in biochemical, biomedical and
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Studies on the protective effect of dietary fish oil on cisplatin induced nephrotoxicity in rats

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Abstract

Cisplatin (CP) is a major antineoplastic drug for the treatment of solid tumors, however, dose dependent nephrotoxicity remains the major concern for its long term use. Several agents/strategies were attempted to prevent CP nephrotoxicity but were not found suitable for clinical practice. Dietary fish oil (FO) enriched in ω-3 fatty acids has been shown to prevent/reduce the progression of certain types of cancers, cardiovascular and renal disorders. The present study was undertaken to see whether FO can prevent CP-induced nephrotoxicity and other deleterious effects. Rats were preed experimental diets for 10 days and then received a single dose of CP (6 mg/kg body weight) intraperitoneally while still on diet. Serum/urine parameters, enzymes of carbohydrate metabolism, brush border membrane (BBM) and oxidative stress in rat kidney were analyzed. CP nephrotoxicity was recorded by increased serum creatinine and blood urea nitrogen. CP decreased the activities of metabolic enzymes, antioxidant defense system and BBM enzymes. In contrast, FO alone increased enzyme activities of carbohydrate metabolism and brush border membrane (BBM). FO feeding to CP treated rats markedly enhanced resistance to CP-elicited deleterious effects. Dietary FO supplementation ameliorated CP induced specific metabolic alterations and oxidative damage due to its intrinsic biochemical antioxidant properties.

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1. Introduction

Cisplatin (cis-diaminedichloroplatinum II, CP) one of the most effective chemotherapeutic agent that plays a major role in the treatment of variety of human solid tumors including those of the head, neck, testis, ovary and breast (Lebwohl and Canetta, 1998). Inspite of its beneficial antitumor action, dose related nephrotoxicity and neurotoxicity limits its application in clinical oncology (Antonio et al., 2002).

Light and electron microscopy have shown that the CP-induced injury and necrosis in the rat kidney are predominantly localized in S3 subsegments of proximal tubular epithelial cells (Townsend et al., 2003). Morphologically, it is characterized by the loss of microvilli, cellular swelling, and condensation of nuclear chromatin (Kuhlmann et al., 1997). Mitochondria, lysosomes and microsomes are critical CP targets (Zhang and Lindup, 1993; Leibbrandt et al., 1995). Functional alterations are characterized by change in urine volume, increase in blood urea nitrogen and serum creatinine (Yao et al., 2007).

The mechanism underlying the side effects induced by cisplatin is not understood clearly, however it was considered to be attributed to the combination of multi-ways (Hong et al., 2005; Ramesh and Reeves, 2002; Nowak, 2002; Townsend and Hanigan, 2002; Xiao et al., 2003) such as the generation of reactive oxygen species (ROS), which could interfere with the antioxidant defense system and result in oxidative damage in different tissues (Kuhlmann et al., 1997; Matsushima et al., 1998; Kuhad et al., 2007; Khan et al., 2009a,b) and reaction with thiols in protein and glutathione, which could cause cell dysfunction.

Various studies have focused on the ways for prevention of cisplatin associated side effects via supplementation of preventive agents simultaneously. These include antioxidants, modulators of nitric oxide, diuretics, and cytoprotective and apoptotic agents (Ali and Al Moundhri, 2006). However, none of these were found to be suitable.

Renewed interest has been observed in recent years in ω-3 fatty acids enriched fish oil (FO) diet that has profound beneficial health effects against various pathologies (Simopoulos, 1991) including...
cardiovascular, respiratory, inflammatory and immune renal diseases; diabetes, depression and cancer (Caterina et al., 1994; Aronson et al., 2001; Donadio, 2001; Hibbenn, 1998; Posta et al., 2006). Previous studies have shown protective role of dietary FO against drug induced nephrotoxicity (Abdel-Gayoum et al., 1995; Thakkar et al., 2000). However, it is important that the prospective nephro protective agent should not affect the drug efficacy while reducing its side effects. There is evidence that dietary supplementation of FO enhances the antitumor effects of CP chemotherapy (Yam et al., 2001). Recent study from our lab has shown that dietary FO supplementation ameliorates GM induced nephrotoxic effects (Priyamvada et al., 2008). However, the protective effect of FO on CP induced nephrotoxicity remains uninvestigated.

Considering the potential clinical use of CP and numerous health benefits of FO, the present work was undertaken to study the biochemical events/cellular response/mechanisms of CP nephropathy and its protection by dietary FO. We hypothesized that fish oil would prevent CP-induced nephrotoxicity due to its intrinsic biochemical and antioxidant properties that would lead to improved metabolism and antioxidant defense mechanism of the kidney. The results obtained indicate that dietary supplementation with fish oil markedly ameliorate CP induced nephrotoxicity parameters and support a potential therapeutic use of CP + FO combination in combating cancer without nephrotoxic and other harmful side effects.

2. Materials and methods

2.1. Chemicals and drugs

Fish oil of menhaden and cisplatin from Sigma Chemical Co., (St. Louis, MO, USA). All other chemicals used were of analytical grade and were purchased either from Sigma Chemical Co. or SRL (Mumbai, India).

2.2. Diet

A nutritionally adequate laboratory pellet diet was obtained from Aashirwaad Industries, Chandigarh (India). Pellets were crushed finely and mixed with 15% fish oil and stored in airtight containers. Vitamin E as α-tocopherol (≥70 mg/kg chow) was added to each of the modified rat chows in order to meet the increased metabolic requirement for antioxidants on a diet high in polyunsaturated fatty acids.

2.3. Experimental design

The animal experiments were conducted according to the guidelines of Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India. Adult male Wistar rats (8 rats/group) weighing between 150 and 200 g were used in the study. Animals were acclimatized to the animal facility for a week on standard rat chow and allowed water ad libitum under controlled conditions of 25 ± 2°C temperature, 50 ± 15% relative humidity and normal photoperiod (12 h dark and light). Four groups of rats entered the study after acclimatization (Fig. 1). They were fed on either normal diet (control and CP groups) or diet containing 15% fish oil (CP and FO groups). After 10 days rats in two groups (CP and CF) were administered a single dose of CP intraperitoneally (6 mg/kg body weight) in 0.9% saline. Animals in the control and FO group received an equivalent amount of normal saline. The rats were sacrificed 4 days after CP administration under light ether anesthesia. Blood and urine samples were collected and kidneys were removed and processed for the preparation of homogenates and BBMV (brush border membrane vesicles) as described below. All the preparations and analyses of various parameters were performed simultaneously under similar experimental conditions to avoid any day to day variations. Body weight of rats were recorded at the start and completion of the experimental procedures.

2.4. Preparation of homogenates

After the completion of the experiment, the kidneys were removed, decapsulated and kept in ice-cold buffered saline (154 mM NaCl, 5 mM Tris-HEPES, pH 7.5). The cortex was carefully separated from medulla as described earlier (Khundmiri et al., 2005). A 15% (w/v) homogenate was prepared in 0.1 M Tris-HCl buffer, pH 7.0 (20 ml of buffer/g of cortical tissue), in a glass Teflon homogenizer with four complete strokes. The homogenate was then subjected to high speed Ultra-Throw homogenizer (Type T-25, Janke & Kunkel GMBH & Co., KG, Staufen) for three strokes of 15 s each with an interval of 15 s between each stroke. MgCl₂ was added to the homogenate to a final concentration of 10 mM and the mixture stirred for 20 min on ice. The homogenate was centrifuged at 1000 x g for 10 min in a Beckman centrifuge (J2 M1, Beckman instruments Inc. Palo Alto, C.A. USA) using JA-17 rotor and the supernatant was then recentrifuged at 35,000 x g for 30 min. The pellet was resuspended in 100 mM mannitol and 5 mM Tris-HEPES, pH 7.4, with four passes by a loose fitting Dounce homogenizer (Wheaton, IL USA) and centrifuged at 35,000 x g for 20 min in a 15 ml corex tube. The outer white fluffy pellet of BBM was resuspended in small volume of buffered 300 mM mannitol. Aliquots of homogenates and BBM were saved and stored at -20°C for BBM enzyme analysis. Each sample of BBM was prepared by pooling tissues from two to three rats.

2.5. Preparation of brush border membrane

BBMV were prepared from whole cortex using the MgCl₂ precipitation method as described previously (Khundmiri et al., 2005). Briefly, freshly minced cortical slices were homogenized in 50 mM mannitol and 5 mM Tris-HEPES buffer, pH 7.0 (20 ml of buffer/g of cortical tissue), in a glass Teflon homogenizer with four complete strokes. The homogenate was then subjected to high speed Ultra-Throw homogenizer (Type T-25, Janke & Kunkel GMBH & Co., KG, Staufen) for three strokes of 15 s each with an interval of 15 s between each stroke. MgCl₂ was added to the homogenate to a final concentration of 10 mM and the mixture stirred for 20 min on ice. The homogenate was centrifuged at 1000 x g for 10 min in a Beckman centrifuge (J2 M1, Beckman instruments Inc. Palo Alto, C.A. USA) using JA-17 rotor and the supernatant was then recentrifuged at 35,000 x g for 30 min. The pellet was resuspended in 100 mM mannitol and 5 mM Tris-HEPES, pH 7.4, with four passes by a loose fitting Dounce homogenizer (Wheaton, IL USA) and centrifuged at 35,000 x g for 20 min in a 15 ml corex tube. The outer white fluffy pellet of BBMV was resuspended in small volume of buffered 300 mM mannitol. Aliquots of homogenates and BBM were saved and stored at -20°C for BBM enzyme analysis. Each sample of BBM was prepared by pooling tissues from two to three rats.

2.6. Serum/urine chemistries

Serum samples were deproteinized with 3% trichloroacetic acid in a ratio of 3:1, left for 10 min and then centrifuged at 2000 x g for 10 min. The protein free supernatant was used to determine inorganic phosphate and creatinine. The precipitate was used to quantify total phospholipids. Blood urea nitrogen (BUN) and cholesterol levels were determined directly in serum samples. Glucose was estimated by a foliuridase method using kit from Span diagnostics, Mumbai, India. All these parameters were determined by standard procedures as mentioned in a previous study (Khundmiri et al., 2004).

Fig. 1. Experimental design (ND: normal diet; C: control; FO: fish oil diet; CP: cisplatin; CPF: fish oil + cisplatin; i.p.: intraperitoneal; CP injection (') normal saline injection (†)).
2.7. Assay of carbohydrate metabolism enzymes

The activities of the enzymes involving oxidation of NADH or reduction of NADP+ were determined spectrophotometrically on a Unicam SP 8000 using 3 ml of assay buffer in 1 cm cuvette at room temperature (28-30 °C). The enzyme activities of lactate dehydrogenase (LDH, EC 1.1.1.27), malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.1.40), glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), glucose-6-phosphatase (G6Pase, EC 3.1.1.31) and fructose-1, 6-bisphosphatase (FBPase, EC 3.1.1.31) were assayed as described by Khundmiri et al. (2004). Hexokinase was measured by the method of Crane and Soh (1955) and the remaining glucose was measured by the method of Nelson-Somogyi (Nelson, 1944).

3. Results

The present work was undertaken to study detailed mechanism of CP-induced nephrotoxicity and other deleterious effects and its possible protection by feeding ω-3 fatty acids enriched fish oil diet to the rats. To address our hypothesis, the effect of CP alone and in combination with fish oil (FO) was determined as described by Farooq et al. (2004).

3.1. Effect of dietary fish oil (FO) on CP induced nephrotoxicity in serum and urine parameters

Results summarized in Tables 1 and 2 show the effect of CP alone and in combination with FO diet on blood and urine chemistry. CP treatment to control rats resulted in significant increase in serum creatinine (Scr) and blood urea nitrogen (BUN), but decrease in cholesterol, phospholipids (PL), inorganic phosphate and glucose compared to control rats. These changes were associated with profound phosphaturia, proteinuria and glucosuria accompanied by decreased creatinine clearance. FO diet alone caused significant increase in serum glucose, Pi and creatinine clearance, decrease in BUN, urinary Pi and protein excretions.

Feeding of FO diet to CP administered (CPF) rats resulted in significant reversal of various CP elicited deleterious effects on serum and urine parameters. FO prevented CP induced increase of Scr, BUN and decrease of serum Pi, PLP and glucose. CP-induced phosphaturia, proteinuria and glucosuria were absent in CPF compared to CP rats.

3.2. Effect of dietary fish oil (FO) on CP induced alterations in biomarker enzymes of BBM and lysosomes

To assess the structural integrity of certain organelles e.g., plasma membrane (BBM) and lysosomes, the effect of CP alone and in combination with FO diet was determined on biomarker enzymes of BBM and lysosomes in the homogenates of renal cortex and medulla and isolated BBM preparations from renal cortex.

3.2.1. Effect of CP alone and with FO diet on biomarkers of BBM and lysosomes in the homogenates (Table 3)

The activities of alkaline phosphatase (ALP), γ-glutamyl transferase (GGTase) and acid phosphatase (ACPase) were determined as described by Farooq et al. (2004). A probability level of p< 0.05 was selected as indicating statistical significance. Most of the changes between various groups were compared with control values for better understanding and clarity.

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Creatinine (mg/dl)</th>
<th>BUN (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>Phospholipid (mg/dl)</th>
<th>Phosphate (µmol/ml)</th>
<th>Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.999±0.046</td>
<td>14.40±0.66</td>
<td>129.2±1.96</td>
<td>114.7±1.86</td>
<td>2.3±0.047</td>
<td>73.2±4.07</td>
</tr>
<tr>
<td>CP</td>
<td>2.012±0.139 (+101.40%)</td>
<td>34.24±33.0 (+137.7%)</td>
<td>115.79±1.29 (-10.37%)</td>
<td>80.68±0.38 (-20.66%)</td>
<td>1.92±0.123 (-16.52%)</td>
<td>19.16±0.99 (-73.83%)</td>
</tr>
<tr>
<td>CPF</td>
<td>1.92±0.123 (-16.52%)</td>
<td>18.25±8.60 (+26.73%)</td>
<td>88.40±1.33 (-31.57%)</td>
<td>98.51±3.58 (-14.12%)</td>
<td>2.0±0.063 (-13.04%)</td>
<td>75.74±3.48 (-34.48%)</td>
</tr>
<tr>
<td>FO</td>
<td>2.3±0.047 (+9.13%)</td>
<td>83.06±5.32 (+13.435)</td>
<td>117.0±6.43 (-18.12%)</td>
<td>99.5±1.97 (-13.25%)</td>
<td>2.5±0.031 (-13.13%)</td>
<td>83.06±5.32 (+13.435)</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for five different preparations. CP: cisplatin treated; CPF: fish oil + cisplatin treated; FO: fish oil diet; BUN: blood urea nitrogen. Values in parentheses represent percent change from control.

3.2.2. Effect of CP and CP plus FO diet on BBM markers in isolated BBMV

The effect of CP and FO on BBM marker enzymes was further analyzed in BBMV preparations isolated from the renal cortex (Table 4). The data shows a similar activity pattern of BBM enzyme activities in cortical homogenates. The feeding of FO diet prior to CP treatment prevented CP elicited decrease in BBM enzyme activities. As can be seen from the data CP induced decrease in BBM enzyme activities were similarly prevented by FO diet. However, the activity of acid phosphatase (ACPase) was increased (+32.15%) by CP in cortical homogenates and FO diet was able to prevent the increase in enzyme activity in a similar manner (Table 3).

The activities of BBM enzymes similar to the cortex were also lowered in the medulla by CP administration (Table 3). The consumption of FO in combination with CP treatment resulted in the reversal of CP induced decrease in ALP (~42.21%), GGTase (~37.10%) and LAP (~23.6%) in the medulla. The activity of ACPase (~35.45%) was increased by CP treatment. However, this increase was prevented by FO feeding to CP treated rats.

Table 4

<table>
<thead>
<tr>
<th>Group</th>
<th>Creatinine (mg/dl)</th>
<th>BUN (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>Phospholipid (mg/dl)</th>
<th>Phosphate (µmol/ml)</th>
<th>Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.999±0.046</td>
<td>14.40±0.66</td>
<td>129.2±1.96</td>
<td>114.7±1.86</td>
<td>2.3±0.047</td>
<td>73.2±4.07</td>
</tr>
<tr>
<td>CP</td>
<td>2.012±0.139 (+101.40%)</td>
<td>34.24±33.0 (+137.7%)</td>
<td>115.79±1.29 (-10.37%)</td>
<td>80.68±0.38 (-20.66%)</td>
<td>1.92±0.123 (-16.52%)</td>
<td>19.16±0.99 (-73.83%)</td>
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<td>CPF</td>
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<td>2.0±0.063 (-13.04%)</td>
<td>75.74±3.48 (-34.48%)</td>
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<td>2.3±0.047 (+9.13%)</td>
<td>83.06±5.32 (+13.435)</td>
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<td>2.5±0.031 (-13.13%)</td>
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</tr>
</tbody>
</table>
33. Effect of fish oil (FO) on CP induced alterations in metabolic enzymes in renal cortex and medulla

The effect of CP, FO diet and their combined treatment was determined on the activities of various enzymes of carbohydrate metabolism in renal cortex and medulla.

3.3. Effect of fish oil (FO) on CP induced alterations in metabolic enzymes in renal cortex and medulla

The effect of CP, FO diet and their combined treatment was determined on the activities of various enzymes of carbohydrate metabolism in renal cortex and medulla.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Urine flow rate (UFR) (ml/h)</th>
<th>Creatinine clearance (ml/min/100 g body weight)</th>
<th>Phosphate (µmol/ml)</th>
<th>Protein (mg/mmol creatinine)</th>
<th>Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18 ± 1.73</td>
<td>0.655 ± 0.002</td>
<td>1.16 ± 0.023</td>
<td>0.029 ± 0.003</td>
<td>13.98 ± 1.73</td>
</tr>
<tr>
<td>CP</td>
<td>24 ± 0.97</td>
<td>0.212 ± 0.097 (−67.63%)</td>
<td>1.65 ± 0.77 (−42.42%)</td>
<td>0.101 ± 0.004 (−255.17%)</td>
<td>56.47 ± 5.51 (−303.93%)</td>
</tr>
<tr>
<td>CPF</td>
<td>21 ± 2.88</td>
<td>0.355 ± 1.0 (−45.81%)</td>
<td>1.19 ± 0.028 (−2.58%)</td>
<td>0.064 ± 0.021 (−20.68%)</td>
<td>13.16 ± 1.60 (−131.19%)</td>
</tr>
<tr>
<td>FO</td>
<td>20 ± 0.57</td>
<td>0.954 ± 0.0012 (−45.64%)</td>
<td>0.644 ± 0.1 (−44.48%)</td>
<td>0.020 ± 0.0 (−31.03%)</td>
<td>4.65 ± 0.715 (−66.66%)</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for five different experiments.

Values in parentheses represent percent change from control.

CP: cisplatin treated; CPF: fish oil + cisplatin treated; FO: fish oil diet.

* Significantly different from control.

### Table 3

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>(a) Cortex</th>
<th>(b) Medulla</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CP</td>
</tr>
<tr>
<td>ALP (µmol/mg protein/h)</td>
<td>7.48 ± 0.54</td>
<td>4.8 ± 0.215 (−35.82%)</td>
</tr>
<tr>
<td>GGTase (µmol/mg protein/h)</td>
<td>10.18 ± 5.91</td>
<td>6.44 ± 1.29 (−36.73%)</td>
</tr>
<tr>
<td>LAP (µmol/mg protein/h)</td>
<td>12.34 ± 1.45</td>
<td>8.64 ± 1.12 (−29.98%)</td>
</tr>
<tr>
<td>ACPase (µmol/mg protein/h)</td>
<td>3.39 ± 0.088</td>
<td>4.48 ± 0.194 (−32.15%)</td>
</tr>
</tbody>
</table>

Results (specific activity expressed as µmol/mg protein/h) are mean ± SEM for five different experiments.

Values in parentheses represent percent change from control.

CP: cisplatin treated; CPF: fish oil + cisplatin treated; FO: fish oil diet; ALP: alkaline phosphatase; GGTase: γ-glutamyl transferase; LAP: leucine aminopeptidase; ACPase: acid phosphatase; BBM: brush border membrane.

* Significantly different from control.

### Table 4

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Groups</th>
<th>Control</th>
<th>CP</th>
<th>CPF</th>
<th>FO</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (µmol/mg protein/h)</td>
<td>45.41 ± 5.53</td>
<td>50.17 ± 1.46 (−54.28%)</td>
<td>78.1 ± 2.49 (−36.84%)</td>
<td>42.74 ± 1.52 (−5.87%)</td>
<td></td>
</tr>
<tr>
<td>GGTase (µmol/mg protein/h)</td>
<td>81.22 ± 4.19</td>
<td>56.28 ± 3.44 (−30.71%)</td>
<td>79.42 ± 2.98 (−36.84%)</td>
<td>94.15 ± 0.815 (−35.19%)</td>
<td></td>
</tr>
<tr>
<td>LAP (µmol/mg protein/h)</td>
<td>66.56 ± 2.5</td>
<td>52.07 ± 2.2 (−21.76%)</td>
<td>66.17 ± 5.55 (−25.58%)</td>
<td>75.38 ± 3.01 (−13.25%)</td>
<td></td>
</tr>
</tbody>
</table>

Results (specific activity expressed as µmol/mg protein/h) are mean ± SEM for five different experiments.

Values in parentheses represent percent change from control.

CP: cisplatin treated; CPF: fish oil + cisplatin treated; FO: fish oil diet; ALP: alkaline phosphatase; GGTase: γ-glutamyl transferase; LAP: leucine aminopeptidase; BBM: brush border membrane vesicles.

* Significantly different from control.

### Tables 5a and 6a

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>(a) Cortex</th>
<th>(b) Medulla</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CP</td>
</tr>
<tr>
<td>ALP (µmol/mg protein/h)</td>
<td>75.38 ± 3.01</td>
<td>66.56 ± 2.5</td>
</tr>
<tr>
<td>GGTase (µmol/mg protein/h)</td>
<td>66.17 ± 5.55</td>
<td>75.38 ± 3.01 (−13.25%)</td>
</tr>
<tr>
<td>LAP (µmol/mg protein/h)</td>
<td>81.22 ± 4.19</td>
<td>56.28 ± 3.44 (−30.71%)</td>
</tr>
</tbody>
</table>

Effect on carbohydrate metabolism in renal cortex

As shown in Tables 5a and 6a, CP treatment to control rats significantly increased the activity of lactate dehydrogenase (LDH) and hexokinase (HK) but decreased malate dehydrogenase (MDH), glucose-6-phosphatase (G6Pase) and fructose-1,6-bisphosphatase (FBPase) activities in the renal cortex. When CP treatment was extended to FO fed rats, CP-induced alterations in metabolic enzyme activities were not only prevented by FO diet, but G6Pase activity remained significantly higher in CPF compared to CP  

### Table 6b

Effect on carbohydrate metabolism in renal medulla

The effect of FO, CP and their combined effect was also determined on glucose-6-phosphate dehydrogenase (G6PDH) and NADP-malic enzyme (ME) source of NADPH needed in various anabolic reactions (Table 6b). CP treatment to control rats significantly decreased G6PDH but increased ME activity. However, CP...
treatment to FO fed rats reduced the CP induced alterations in glucose-6-phosphate dehydrogenase (G6PDH) and NADP-malic enzyme (ME) activities.

3.3.2. Effect on carbohydrate metabolism in renal medulla

The activities of LDH, MDH, HK, G6Pase and FBPase in medullary homogenates were similarly affected by CP treatment (Tables 5b and 6b) as in the cortical homogenates. Feeding of FO to CP treated rats prevented CP induced alterations in various enzyme activities. Similar to cortex, G6PDH activity decreased whereas ME activity increased by CP treatment in the medulla (Table 6b). Dietary supplementation of FO to CP treated rats decreased G6PDH and ME activity.

3.4. Effect of dietary fish oil (FO) on CP induced alterations in antioxidant defense parameters in renal cortex and medulla

It is evident that reactive oxygen species generated by various toxicants are important mediators of cellular injury and pathogenesis of various diseases (Walker et al., 1999). Antioxidant status is a potential biomarker to determine the physiological state of the cell, tissue or organ. To ascertain the role of antioxidant system in CP-induced toxicity, the effect of CP was observed on oxidative stress parameters. CP enhanced lipid peroxidation (LPO) and significantly altered antioxidant enzymes both in cortex and medulla, albeit differently (Table 7). LPO measured in terms of malondialdehyde (MDA) levels was significantly enhanced in the cortex (+63.73%) and medulla (+28.9%), whereas total-SH declined in these tissue (−30.36%) and (−33.33%), respectively. CP treatment caused significant decrease in superoxide dismutase (SOD, −45.69%), glutathione peroxidase (GSH-Px, −40.74%) and catalase (−19.96%) activities in the renal cortex. In medulla, however the activity of SOD (−51.98%), catalase (−59.15%) and GSH-Px (−38.46%) were profoundly decreased by CP administration alone. However, FO consumption significantly enhanced antioxidant parameters. CP administration to FO fed rats prevented the decline in superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase. The results indicate marked protection by FO diet against CP induced oxidative damage to renal tissues.

4. Discussion

Cisplatin is an effective chemotherapeutic agent that is widely used in the treatment of many malignant tumors, including head
Table 6
Effect of fish oil (FO) on activities of G6Pase, FBPase, G6PDH and ME in homogenates of (a) cortex and (b) medulla with/without CP treatment.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Groups</th>
<th>Control</th>
<th>CP</th>
<th>CPF</th>
<th>FO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(μmol/mg protein/h)</td>
<td>(μmol/mg protein/h)</td>
<td>(μmol/mg protein/h)</td>
<td>(μmol/mg protein/h)</td>
</tr>
<tr>
<td>Cortex</td>
<td>G6Pase</td>
<td>1.91 ± 0.121</td>
<td>1.536 ± 0.046</td>
<td>1.83 ± 0.101</td>
<td>2.46 ± 0.037</td>
</tr>
<tr>
<td></td>
<td>FBPase</td>
<td>3.44 ± 0.068</td>
<td>2.05 ± 0.157</td>
<td>2.26 ± 0.095</td>
<td>2.50 ± 0.020</td>
</tr>
<tr>
<td></td>
<td>G6PDH</td>
<td>0.288 ± 0.0065</td>
<td>0.195 ± 0.011</td>
<td>0.223 ± 0.024</td>
<td>0.295 ± 0.013</td>
</tr>
<tr>
<td></td>
<td>ME</td>
<td>0.215 ± 0.009</td>
<td>0.266 ± 0.013</td>
<td>0.245 ± 0.006</td>
<td>0.226 ± 0.009</td>
</tr>
</tbody>
</table>

(b) Medulla

<table>
<thead>
<tr>
<th>Control</th>
<th>CP</th>
<th>CPF</th>
<th>FO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>2.83 ± 0.104</td>
<td>2.73 ± 0.237</td>
<td>2.53 ± 0.127</td>
</tr>
<tr>
<td>Medulla</td>
<td>5.82 ± 0.635</td>
<td>3.64 ± 0.301</td>
<td>3.76 ± 0.205</td>
</tr>
</tbody>
</table>

Results (specific activity expressed as μmol/mg protein/h) are mean ± SEM for five different experiments.

Values in parentheses represent percent change from control.

CP: cisplatin treated; CPF: fish oil + cisplatin treated; FO: fish oil diet; G6Pase: glucose-6-phosphatase; FBPase: fructose-1,6-bisphosphatase; G6PDH: glucose-6-phosphatase dehydrogenase; ME: malic enzyme.

Table 7
Effect of fish oil (FO) on enzymatic and non enzymatic antioxidant parameters in homogenates of (a) cortex and (b) medulla with/without CP treatment.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Lipid peroxidation (nmol/g tissue)</th>
<th>Total SH (μmol/g tissue)</th>
<th>SOD (μmol/mg protein)</th>
<th>Catalase (μmol/mg protein/min)</th>
<th>GSH-Px (μmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Cortex</td>
<td>Control</td>
<td>94.61 ± 12.92</td>
<td>15.38 ± 0.125</td>
<td>25.67 ± 0.035</td>
<td>76.13 ± 1.01</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>154.91 ± 9.2 (63.73%)</td>
<td>10.71 ± 0.237 (30.38%)</td>
<td>13.94 ± 1.106 (45.96%)</td>
<td>60.93 ± 1.18 (49.96%)</td>
</tr>
<tr>
<td></td>
<td>CPF</td>
<td>111.79 ± 5.42 (18.15%)</td>
<td>13.07 ± 0.751 (15.01%)</td>
<td>12.97 ± 0.905 (14.02%)</td>
<td>71.33 ± 1.44 (15.15%)</td>
</tr>
<tr>
<td></td>
<td>FO</td>
<td>102.11 ± 2.6 (76.2)</td>
<td>15.03 ± 1.53 (2.27%)</td>
<td>15.58 ± 2.22 (38.5%)</td>
<td>83.55 ± 1.17 (94.74%)</td>
</tr>
<tr>
<td>(b) Medulla</td>
<td>Control</td>
<td>27.3 ± 1.72</td>
<td>1.47 ± 0.078</td>
<td>25.16 ± 1.73</td>
<td>97.66 ± 3.43</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>35.19 ± 1.72 (28.9%)</td>
<td>0.98 ± 0.023 (33.33%)</td>
<td>12.08 ± 0.557 (51.98%)</td>
<td>38.99 ± 2.36 (39.15%)</td>
</tr>
<tr>
<td></td>
<td>CPF</td>
<td>33.60 ± 1.34 (23.07%)</td>
<td>1.05 ± 0.061 (92.88%)</td>
<td>15.32 ± 0.935 (5.32%)</td>
<td>46.09 ± 2.76 (42.56%)</td>
</tr>
<tr>
<td></td>
<td>FO</td>
<td>31.27 ± 0.25 (14.54%)</td>
<td>1.29 ± 0.100 (12.24%)</td>
<td>24.62 ± 1.19 (7.53%)</td>
<td>111.69 ± 0.715 (14.36%)</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for five different experiments.

Values in parentheses represent percent change from control.

CP: cisplatin treated; CPF: fish oil + cisplatin treated; FO: fish oil diet; LPO: lipid peroxidation; total SH: sulphydryl groups; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase.

* Significantly different from CP.

Significantly different from control.
massive proteinuria, glucosuria and phosphaturia. The feeding of FO prior to and after CP treatment significantly lowered CP-elicited increased levels of both serum creatinine and BUN. Serum glucose and phospholipids were improved upon CP treatment to FO consuming rats.

Since the BBM of renal proximal tubules has been shown as the major site of CP-induced renal injury (Arany et al., 2003; Fatima et al., 2004), the integrity of the membrane was assessed by the status of its biomarker enzymes ALP, GGTase and LAP. CP caused significant decrease in the activities of ALP, GGTase and LAP in the cortical homogenates as well as in isolated BBM vesicles. A similar decrease was observed in medulla, suggesting an overall CP induced damage to the kidney. The decrease in BBM enzyme activities might have occurred due to loss of BBM enzymes and other proteinic components from damaged BBM into the lumen that later appear in urine as reported earlier (Khan et al., 2009a,b; Banday et al., 2008; Fatima et al., 2004). In contrast to CP, FO consumption, however, enhanced the activities of BBM enzymes in homogenate and BBM, indicating an overall improvement in renal BBM integrity. FO consumption in combination with CP treatment prevented/retarded CP-induced decrease of BBM enzyme activities in the renal tissues. CP is shown to be metabolized to a nephrotoxin by renal proximal tubule cells and the BBM marker enzyme, GGTase plays an essential role in the pathway involved (Townsend et al., 2003). Inhibition of GGTase activity has been shown to block CP toxicity (Townsend et al., 2003; Townsend and Hanigan, 2002). We speculate that FO might be inhibiting the activity of an essential enzyme in this pathway thereby preventing formation of the nephrotoxin and hence reducing CP nephrotoxicity. The activity of GGTase was measured in all the experimental groups. GGTase activity was found to increase in FO fed rats while it decreased in CP treated rats as compared to control suggesting that FO might be ameliorating the effect of CP by inhibiting another enzyme and not GGTase crucial/essential for the pathway involved in metabolic activation of CP to a nephrotoxin. The activity of lysosomal enzyme, ACPase was significantly increased in the cortex and medulla by CP treatment. Alteration in ACPase activity demonstrates CP-induced loss of lysosomal function (Kuhlmann et al., 1997; Courjault-Gautier et al., 1995).

Since the major function of kidney is to reabsorb important ions and molecules, that interun depends on the structural integrity of BBM and available energy as ATP which is supplied by FO consumption. CP caused significant increase in LDH and decrease in MDH in the renal cortex and medulla, which was associated with simultaneous increase in hexokinase activity in the renal tissues. Although the actual rates of glycolysis or TCA cycle were not determined, marked decrease in MDH activity appears to be due to CP-induced damage to mitochondria (Kuhlmann et al., 1997; Zhang and Lindup, 1993). A marked increase in LDH and to some extent hexokinase activity with simultaneous decline in TCA cycle enzyme, MDH appears to be an adaptive cellular effect in energy metabolism from aerobic metabolism disturbance of various cell organelles (Kuhlmann et al., 1997). Different studies have shown that cytotoxicity of CP is probably due to combination of insults including peroxidation of cell membrane, mitochondrial dysfunction, inhibition of protein synthesis and DNA damage in the kidney (Santos et al., 2007). It has been suggested that CP induces renal damage by free radical generation, such as hydroxyl radical and superoxide anion, by altering arginine metabolism, by increasing activity of calcium independent nitric oxide synthase and more recently by apoptosis (Santos et al., 2007). The feeding of FO diet to CP treated rats prevented CP induced augmentation of LPO and suppression of antioxidant enzyme activities. The protection against CP effect by FO can be attributed to its intrinsic biochemical and natural antioxidant properties. Thus, it appears that FO enriched in ω-3 fatty acids enhanced resistance to free radical attack generated by CP administration similarly as demonstrated in lupus nephritis and other pathologies (Chandraseka and Fernandez, 1994; Xi and Chen, 2000; Donadini, 2001).

Although the mechanism involved in cisplatin nephrotoxicity have been extensively studied, they are not yet fully elucidated. It has been shown that CP induces necrosis of the S3 subsegment of proximal tubule (Townsend et al., 2003; Portilla et al., 2006). CP has been shown to be activated to a potent nephrotoxin in renal proximal tubules (Townsend et al., 2003). CP nephrotoxicity has been morphologically characterized by loss of microvilli, alteration in the number and size of lysosomes and mitochondrial vacuolization accompanied by functional disturbances of various cell organelles (Kuhlmann et al., 1997). Different studies have shown that cytotoxicity of CP is probably due to combination of insults including peroxidation of cell membrane, mitochondrial dysfunction, inhibition of protein synthesis and DNA damage in the kidney (Santos et al., 2007). It has been suggested that CP induces renal damage by free radical generation, such as hydroxyl radical and superoxide anion, by altering arginine metabolism, by increasing activity of calcium independent nitric oxide synthase and more recently by apoptosis (Santos et al., 2007; Devipriya and Shyamaladevi, 1999; Arany et al., 2004). However, CP-induced oxidative stress was shown to be strongly involved in acute renal damage (Gonzales et al., 2005). The present findings at least in part support above proposed mechanism. To summarize, our results (Fig. 3) indicate an overall improvement in metabolic activities, BBM integrity and antioxidant defenses upon dietary FO supplementation to CP treated rats.
5. Conclusion

The present results show that CP elicited deleterious nephrotoxic and other adverse effects as indicated by significant increase in serum creatinine, BUN, decrease in the activities of various enzymes of BBM, mitochondria and metabolism. The effects appeared to be mediated, at least, in part due to CP-induced perturbation in antioxidant defense mechanism. FO consumption to a larger extent prevented CP-induced nephrotoxicity parameters by enhancing energy metabolism, BBM enzymes and by strengthening antioxidant defense mechanism. Taking into account these results, we propose that FO may maximize the clinical use of CP in the treatment of various malignancies without nephrotoxic and other side effects.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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References


Studies on the protective effect of flaxseed oil on cisplatin-induced hepatotoxicity

A Naqshbandi, W Khan, S Rizwan and F Khan

Abstract
Cisplatin (CP) is known as one of the most potent chemotherapeutic antitumor drugs. The tissue-specific toxicity of CP in the kidneys is well documented. However, at higher doses less common toxic effects such as hepatotoxicity may arise. Since CP remains one of the most effective antineoplastic drug used in chemotherapy, strategies to protect tissues against CP toxicity are of clinical interest. Recently, ω-3 polyunsaturated fatty acids (PUFAs) from certain plants/seeds notably flaxseed have shown numerous health benefits. In view of this, the present study investigates the protective effect of flaxseed oil (FXO) on CP-induced damage in liver. Rats were pre-fed normal diet and the diet rich in FXO for 10 days and then a single dose of CP (6 mg/kg body weight) was administered intraperitoneally while still on diet. Serum/urine parameters, enzymes of carbohydrate metabolism and oxidative stress were analyzed. CP caused perturbation of the antioxidant defense as reflected by the decrease in the activities of catalase, superoxide dismutase and glutathione peroxidase. Further the activities of various enzymes involved in glycolysis, tricarboxylic acid cycle, gluconeogenesis and hexose monophosphate shunt pathways were determined and were found to be differentially altered by CP treatment. However, these alterations were ameliorated in CP-treated rats fed on FXO. Present results show that dietary supplementation of FXO in CP-treated rats ameliorated CP-induced hepatotoxic and other deleterious effects due to its intrinsic biochemical/antioxidant properties.

Keywords
Cisplatin; hepatotoxicity; flaxseed oil; carbohydrate metabolism; oxidative stress

Introduction
Cisplatin (cis-diamminedichloroplatinum II (CP)) is one of the most effective and widely used chemotherapeutic agents in the treatment of a variety of human solid tumors. The CP-induced ototoxicity and nephrotoxicity have been very well studied in both clinical and animal research, however hepatotoxicity has been rarely paid attention to. Recent studies have suggested that hepatotoxicity is also a major dose-limiting side effect in CP-based chemotherapy. Continued aggressive high-dose CP chemotherapy necessitates the investigation of ways for prevention of the dose-limiting side effects that inhibit the CP administration at tumoricidal doses. CP chemotherapy induces a fall in plasma antioxidant levels, which may reflect a failure of the antioxidant defense mechanism against oxidative damage induced by commonly used antitumor drugs. A relationship between oxidative stress and CP toxicity has been suggested in many experimental models. Several reports have implicated free radicals and reactive oxygen species (ROS) in the toxicity of CP. CP-induced toxicity is closely associated with an increase in lipid peroxidation (LPO), decreased levels of protein bound sulphydryl groups and glutathione which indicate the formation of ROS.

Until now a large number of studies have been focused on the ways for prevention of CP side effects...
via supplementation of preventive agents simultaneously. The past 3 decades have been a period of rapid expansion in the scientific knowledge of ω-3 PUFAs. The most common way to consume ω-3 fatty acids has been in the form of marine oils like fish. Studies from our laboratory have shown that dietary fish oil (FO) supplementation ameliorates gentamicin and CP-induced damage to kidney and liver, respectively. Vegetable sources including grains and oils offer an alternative source for those who are unable to regularly consume fish for religious or other reasons. Flaxseed (Linum usitatissimum) is the richest dietary sources of ω-3 among plant sources. Recently flaxseed has been identified as a significant alternative source of ω-3 fatty acids. Dietary supplementation with flaxseed oil (FXO) affects the biochemistry of fatty acid metabolism and thus the balance of proinflammatory mediators and atherogenic lipids, holding a great promise for modulating inflammatory diseases. FXO has also been shown to increase the life span of irradiated mice, suggesting its prophylactic potential against radiation-induced degenerative changes in liver. However, the protective effect of FXO on CP-induced toxicity has not yet been investigated.

Taking into consideration the potential clinical use of CP and numerous health benefits of FXO, the present work was undertaken to study the detailed biochemical events/cellular response/mechanisms of CP-induced hepatotoxicity and its protection by dietary FXO. We hypothesized that FXO would prevent CP-induced hepatotoxicity due to its intrinsic biochemical and antioxidant properties that would lead to improved metabolism and antioxidant defense mechanism of the liver. The results obtained indicate that dietary supplementation with FXO markedly ameliorated CP-induced adverse effects in liver. The activities of carbohydrate metabolism, membrane and antioxidant enzymes were markedly enhanced by FXO feeding to CP-administered rats. These studies support a potential therapeutic use of CP + FXO combination in combating cancer without hepatotoxic and other harmful side effects.

Materials and methods

Chemicals and drugs

Flaxseed oil: Omega Nutrition Canada Inc., Cisplatin (Sigma Chemical Co., USA). All other chemicals used were of analytical grade and were purchased either from Sigma Chemical Co. (St Louis, MO, USA) or from SRL, Mumbai, India.

Diet

A nutritionally adequate laboratory pellet diet was obtained from Aashirwaad Industries, Chandigarh (India). Pellets were crushed finely and mixed with 15% FXO and stored in airtight containers. Vitamin E as dl-α-tocopherol (270 mg/kg chow) was added to each of the modified rat chows in order to meet the increased metabolic requirement for antioxidants on a diet high in PUFAs.

Experimental design

The animal experiments were conducted according to the guidelines of Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India. Adult male Wistar rats (8 rats/group) weighing between 150 and 200 g were used in the study. Animals were acclimatized to the animal facility for a week on standard rat chow and allowed water ad libitum under controlled conditions of 25 ± 2°C temperature, 50 ± 15% relative humidity and normal photoperiod (12 h dark and light). Four groups of rats entered the study after acclimatization (Figure 1). They were fed on either normal diet (control and CP groups) or diet containing 15% FXO (CPFXO and FXO groups). After 10 days, rats in 2 groups (CP and CPFXO) were administered a single dose of CP intraperitoneally (6 mg/kg body weight) in 0.9% saline. Animals in the control and FXO group received an equivalent amount of normal saline. The rats were killed 4 days after CP administration under light ether anesthesia. Blood and urine samples were collected and liver was removed and processed for the preparation of homogenate as described below. All the preparations and analyses of various parameters were performed simultaneously under similar experimental conditions to avoid any day-to-day variations. Body weights of rats were recorded at the start and completion of the experimental procedures.

Preparation of homogenates

After the completion of treatment schedule, animals were killed, liver was carefully removed from the treated and control animals and homogenized in 0.1 M Tris-HCl buffer (pH 7.5) by a glass-teflon homogenizer (Thomas PA, USA) by passing 5 pulses; at 4°C to make a 10% (w/v) homogenate. The homogenate was then subjected to high-speed Ultra-Turrex Kunkel homogenizer (Type T-25, Janke & Kunkel
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GMBH & Co. KG. Staufen, Germany) for 3 strokes of 30 s each with an interval of 30 s between each stroke. Homogenate was centrifuged at 2000 r/min at 4°C for 10 min in Beckman J2-M1 (Beckman instruments, Inc Palo Alto, CA, USA) high-speed refrigerated centrifuge to remove the cell debris. The supernatant was transferred to aliquots and stored at −20°C for various enzyme activities.

Serum/urine chemistries

Serum samples were deproteinated with 3% trichloroacetic acid in a ratio of 1:3, left for 10 min and then centrifuged at 2000g for 10 min. The protein free supernatant was used to determine inorganic phosphate (Pi) and creatinine. The precipitate was used to quantitate total phospholipids (PLs). Blood urea nitrogen (BUN) and cholesterol levels were determined directly in serum samples. These parameters were determined by standard procedures as mentioned in a previous study. Transaminases, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated by Reitman and Frankel method using kit from span diagnostics. Glucose was estimated by o-toluidene method using kit from Span diagnostics, Mumbai, India.

Assay of carbohydrate metabolism enzymes

The activities of the enzymes involving oxidation of nicotinamide adenine dinucleotide or reduction of nicotinamide adenine dinucleotide phosphate (NADP) were determined spectrophotometrically on Cintra 5 fixed for 340 nm using 3 ml of assay in a 1-cm cuvette at room temperature (28–30°C). The enzyme activities of lactate dehydrogenase (LDH, EC 1.1.1.27), malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.1.40), glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), glucose-6-phosphatase (G6Pase, EC 3.1.3.1) and fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11) were assayed as described by Khundmiri et al. Hexokinase (HK) was estimated by the method of Crane and Sols and the remaining glucose was measured by method of Nelson.

Assay of membrane enzymes and lysosomal marker enzyme

The activities of membrane marker enzymes, alkaline phosphatase (ALP), leucine amino peptidase (LAP), γ-glutamyl transferase (GGTase) and lysosomal marker enzyme, acid phosphatase (ACPase) were determined as described by Farooq et al.

Assay of enzymes involved in free radical scavenging

Superoxide dismutase (SOD, EC 1.15.1.1) was assayed by the method of Marklund and Marklund. Catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (GSH-Px, EC 1.11.1.9) activities were assayed.

Figure 1. Experimental design (ND: normal diet, C: control, FXO: flaxseed oil diet, CP: cisplatin, CPFXO: flaxseed oil + cisplatin treated, i.p.: intraperitoneal; CP injection (): normal saline injection (i)).
by the method of Giri et al. and Flohe and Gunzler, respectively.

**Lipid peroxidation and total –SH group estimation**

Total SH groups were determined by the method of Sedlak and Lindsay and LPO by the method of Ohkawa et al. 

**Statistical analyses**

All data are expressed as mean ± SEM for at least 4-5 different preparations. Statistical evaluation was conducted by one-way analysis of variance (ANOVA) using Origin 6.1 software. A probability level of $p < 0.05$ was selected as indicating statistical significance. Most of the changes between various groups were compared with control values for better understanding and clarity.

**Results**

The present work was undertaken to study detailed mechanism of CP-induced hepatotoxicity and other deleterious effects and its possible protection by feeding ω-3 fatty acids–enriched diet to the rats. To address our hypothesis, the effect of CP alone and in combination with FXO, a rich source of ω-3 fatty acids, was determined on various enzymatic and nonenzymatic parameters of oxidative stress and enzymes of carbohydrate metabolism in rat liver.

**Effect of dietary FXO oil on CP-induced alterations in serum and urinary parameters**

Results summarized in Tables 1 and 2 show the effect of CP alone and in combination with FXO on blood and urine chemistries.

The liver dysfunction induced by CP was characterized by the elevated levels of serum transaminases, ALT and AST. CP treatment to control rats resulted in significant increase in serum transaminases (ALT, +106% and AST, +154%), creatinine (Scr, +176%), BUN (+122.31%) and PL (+77%) but decrease in Pi (–17%) and glucose (–79%) compared to control rats. These changes were associated with profound phosphaturia, proteinuria and glucosuria accompanied by decreased creatinine clearance. FXO diet alone caused decrease in Scr, cholesterol and PL accompanied by significant increase in creatinine clearance.

Feeding FXO diet prior to and following CP administration resulted in significant reversal of various CP-elicited deleterious effects on serum and urine parameters. FXO prevented CP-induced increase in transaminases, Scr and BUN.

**Effect of dietary FXO on CP-induced alterations in membrane enzymes and marker enzyme of lysosomes**

To assess the structural integrity of plasma membrane and lysosomes, the effect of CP alone and in combination with FXO diet was determined on membrane and lysosomal enzymes in the liver homogenate. The activities of membrane marker enzymes viz ALP, GGTase and LAP and lysosomal marker enzyme, ACPase, were determined under different experimental conditions in the homogenates of liver (Figure 2; Table 3). CP treatment to control rats caused significant reduction in the specific activities of ALP (–64%), GGTase (–39%) and LAP (–37%) in liver homogenate. CP treatment to FXO fed rats prevented CP elicited decrease in membrane enzyme activities. As can be seen from the data, CP-induced decrease in enzyme activities were prevented by FXO diet. However, the activity of ACPase was increased (+54%) by CP treatment in liver homogenate, while FXO diet was able to prevent the increase in ACPase activity (Table 3).

**Effect of FXO on CP-induced alterations on metabolic enzymes in liver**

The effect of CP, FXO diet and their combined treatment was determined by the activities of various enzymes of carbohydrate metabolism in liver. As shown in Table 4, CP treatment to control rats significantly increased the activities of LDH (+104.76%), HK (+18%) and ME (+52%) but decreased the activities of MDH (–33%), G6Pase (–22), FBPase (–44%) and G6PDH (–49%). In contrast, FXO consumption enhanced the activities of FBPase and G6Pase. Feeding FXO to CP-treated rats arrested CP-induced decline in MDH, FBPase, G6Pase, G6PDH activities and enhancement in LDH, HK and ME activities.

**Effect of dietary FXO on CP-induced alterations in antioxidant defense parameters in liver**

It is evident that ROS generated by various toxicants are important mediators of cellular injury and
pathogenesis of various diseases. Antioxidant status is a potential biomarker to determine the physiological state of the cell, tissue or organ. To ascertain the role of antioxidant system in CP-induced toxicity, the effect of CP was observed on oxidative stress parameters. CP enhanced LPO and significantly altered antioxidant enzymes albeit differently (Table 5). LPO measured in terms of malondialdehyde levels significantly enhanced in the (+52%), whereas total -SH declined in the liver (−30%) in CP group. CP treatment caused decrease in SOD (−15%), GSH-Px (−76%) and CAT (−69%) activities. In contrast, FXO consumption increased the activities of antioxidant enzymes albeit to different extents. However, when CP treatment was extended to FXO fed rats (CPFXO group), the decline in SOD, GSH-Px and CAT activities was reduced significantly in comparison to CP group. The results indicate marked protection by FXO diet against CP-induced oxidative damage to renal tissues.

Discussion

CP is a major antineoplastic drug used for the treatment of various forms of cancers. The efficacy of CP is limited, however, by its dose-limiting nephrotoxicity. Although CP-induced nephrotoxicity has been very well documented in clinical oncology, hepatotoxicity has been rarely characterized and is less studied. It is known that CP is significantly taken up in human liver and that high doses of the drug produces hepatotoxicity. The treatment of tumor cells with CP provokes several responses including membrane peroxidation, dysfunction of mitochondria, inhibition of protein synthesis and DNA damage. Formation of free radicals leading to oxidative stress has been shown to be one of the pathogenic mechanisms of the adverse effects of CP in kidney and liver. CP is known to cause histopathological and ultrastructural abnormalities in the liver including inflammatory infiltration, hyperplasia, perportal fibrosis, marked disruption of hepatic cords and dilated blood sinuoids. Many hepatocytes showed karyomegaly and pyknotic nuclei indicating apoptosis. Several strategies and agents were utilized to prevent CP-induced toxicity but were not found suitable for clinical practice.

The last 3 decades have witnessed a major drift in the scientific community toward the roles of naturally occurring dietary substances for their ability to confer health and physiological benefits. A number of studies have revealed that ω-3 PUFAs have numerous health benefits. The predominant dietary sources of
Table 1. Effect of flaxseed oil (FXO) on serum parameters with or without CP treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>Creatinine (mg/dl)</th>
<th>BUN (mg/dl)</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
<th>Cholesterol (mg/dl)</th>
<th>Phospholipid (mg/dl)</th>
<th>Phosphate (µmol/l)</th>
<th>Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.942 ± 0.080</td>
<td>16.49 ± 1.16</td>
<td>28.39 ± 0.58</td>
<td>62.32 ± 3.88</td>
<td>51.27 ± 0.66</td>
<td>23 ± 2.3</td>
<td>3.59 ± 0.11</td>
<td>73.51 ± 0.01</td>
</tr>
<tr>
<td>CP</td>
<td>2.60 ± 0.142b</td>
<td>36.66 ± 0.70b</td>
<td>58.52 ± 3.74b</td>
<td>158.4 ± 13.8b×</td>
<td>58.08 ± 1.86b</td>
<td>40.82 ± 1.15b</td>
<td>2.97 ± 0.150</td>
<td>15.21 ± 2.79b</td>
</tr>
<tr>
<td>CPFXO</td>
<td>1.13 ± 0.032c</td>
<td>30.42 ± 1.45b</td>
<td>47.36 ± 1.91b×</td>
<td>143 ± 4.9b×</td>
<td>42.62 ± 2.33c</td>
<td>54.33 ± 0.86b×</td>
<td>3.03 ± 0.025b</td>
<td>36.67 ± 1.45b×</td>
</tr>
<tr>
<td>FXO</td>
<td>0.686 ± 0.018b</td>
<td>19.64 ± 0.714</td>
<td>31.09 ± 1.49</td>
<td>80.29 ± 3.61b</td>
<td>3.79 ± 0.438b</td>
<td>16.96 ± 0.29b</td>
<td>3.28 ± 0.114</td>
<td>64.77 ± 0.30b</td>
</tr>
</tbody>
</table>

CP: cisplatin treated; CPFXO: flaxseed oil + cisplatin treated; FXO: flaxseed oil diet; BUN: blood urea nitrogen; ALT: alanine aminotransferases; AST: aspartate aminotransferases.

*Results are mean ± SEM for five different experiments.

bSignificantly different from control at p < 0.05 by one-way ANOVA. Values in parentheses represent percent change from control.

Table 2. Effect of flaxseed (FXO) on urine parameters with/without CP treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urine flow rate (UFR; ml/day)</th>
<th>Creatinine clearance (ml/min/100 g bwt)</th>
<th>Phosphate (µmol/ml)</th>
<th>Protein (mg/mmol creatinine)</th>
<th>Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.4 ± 1.15</td>
<td>0.22 ± 0.001</td>
<td>0.462 ± 0.005</td>
<td>1.25 ± 0.026</td>
<td>22.37 ± 1.73</td>
</tr>
<tr>
<td>CP</td>
<td>18 ± 1.73b (+114%)</td>
<td>0.032 ± 0.011b (-85%)</td>
<td>0.680 ± 0.115b (+47%)</td>
<td>1.97 ± 0.005b (+58%)</td>
<td>104.1 ± 0.0057b (+365%)</td>
</tr>
<tr>
<td>CPFXO</td>
<td>13.2 ± 0.57b±c (+57%)</td>
<td>0.067 ± 0.017b (-70%)</td>
<td>0.595 ± 0.01b (+29%)</td>
<td>1.78 ± 0.23b (-42%)</td>
<td>65.75 ± 0.28b¹c (+194%)</td>
</tr>
<tr>
<td>FXO</td>
<td>6 ± 0.115b (-29%)</td>
<td>0.258 ± 0.0023b (+17%)</td>
<td>0.261 ± 0.001b (-43%)</td>
<td>0.740 ± 0.115b (-41%)</td>
<td>35.61 ± 0.57b⁰ (+59%)</td>
</tr>
</tbody>
</table>

CP: cisplatin treated; CPFXO: flaxseed oil + cisplatin treated; FXO: flaxseed oil diet.

bResults are mean ± SEM for five different experiments. Values in parentheses represent percent change from control.

bSignificantly different from control at p < 0.05 by one-way ANOVA.

bSignificantly different from CP at p < 0.05 by one-way ANOVA.
Table 3. Effect of flaxseed oil (FXO) on biomarker enzymes of membrane and lysosomes with/without CP treatment in liver homogenates

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Group</th>
<th>ALP (μmol/mg protein/h)</th>
<th>GGTase (μmol/mg protein/h)</th>
<th>LAP (μmol/mg protein/h)</th>
<th>ACPase (μmol/mg protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>5.18 ± 0.430</td>
<td>2.41 ± 0.1</td>
<td>12.44 ± 0.231</td>
<td>1.40 ± 0.025</td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>1.85 ± 0.058b (−64%)</td>
<td>1.47 ± 0.032b (−39%)</td>
<td>7.89 ± 0.35b (−37%)</td>
<td>2.16 ± 0.08b (+54%)</td>
</tr>
<tr>
<td></td>
<td>CPFXO</td>
<td>2.93 ± 0.262bc (−43%)</td>
<td>1.87 ± 0.03bc (−22%)</td>
<td>10.78 ± 0.19bc (−13%)</td>
<td>1.77 ± 0.109b (+26%)</td>
</tr>
<tr>
<td></td>
<td>FXO</td>
<td>5.17 ± 0.241 (−16%)</td>
<td>3.70 ± 0.168b (−57%)</td>
<td>13.98 ± 0.27b (−12%)</td>
<td>1.23 ± 0.092 (−12%)</td>
</tr>
</tbody>
</table>

CP: cisplatin treated; CPFXO: flaxseed oil + cisplatin treated; FXO: flaxseed oil diet; ALP: alkaline phosphatase; GGTase: γ-glutamyl transferase; LAP: leucine aminopeptidase; ACPase: acid phosphatase.

*Results (specific activity expressed as μmoles/mg protein/h) are mean ± SEM for five different experiments. Values in parentheses represent percent change from control.

bSignificantly different from control at p < 0.05 by one-way ANOVA.

Table 4. Effect of flaxseed oil (FXO) on carbohydrate metabolic enzymes with/without CP treatment in liver homogenates

<table>
<thead>
<tr>
<th>Group</th>
<th>LDH (μmol/mg protein/h)</th>
<th>MDH (μmol/mg protein/h)</th>
<th>HK (mmol/mg protein/h)</th>
<th>G6Pase (mmol/mg protein/h)</th>
<th>FBPase (mmol/mg protein/h)</th>
<th>ME (mmol/mg protein/h)</th>
<th>G6PDH (mmol/mg protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.05 ± 0.042</td>
<td>1.46 ± 0.1</td>
<td>1.08 ± 0.07</td>
<td>0.339 ± 0.004</td>
<td>2.13 ± 0.07</td>
<td>0.021 ± 0.002</td>
<td>0.116 ± 0.0028</td>
</tr>
<tr>
<td>CP</td>
<td>2.15 ± 0.09b (−33%)</td>
<td>0.985 ± 0.004b</td>
<td>1.27 ± 0.215</td>
<td>0.264 ± 0.003b</td>
<td>1.19 ± 0.069b</td>
<td>0.032 ± 0.021b</td>
<td>0.059 ± 0.002b</td>
</tr>
<tr>
<td></td>
<td>(+105%)</td>
<td></td>
<td>(-18%)</td>
<td>(-22%)</td>
<td>(-44%)</td>
<td>(-52%)</td>
<td>(-49%)</td>
</tr>
<tr>
<td>CPFXO</td>
<td>0.823 ± 0.023b (−22%)</td>
<td>1.16 ± 0.037b (−21%)</td>
<td>1.19 ± 0.051</td>
<td>0.287 ± 0.047</td>
<td>1.57 ± 0.06b (−15%)</td>
<td>0.024 ± 0.05e (−14%)</td>
<td>0.082 ± 0.019b (−29%)</td>
</tr>
<tr>
<td></td>
<td>(+64%)</td>
<td></td>
<td>(-10%)</td>
<td>(−26%)</td>
<td>(−14%)</td>
<td>(−29%)</td>
<td>(−29%)</td>
</tr>
<tr>
<td>FXO</td>
<td>0.447 ± 0.06b (−57%)</td>
<td>1.44 ± 0.130</td>
<td>0.98 ± 0.036</td>
<td>0.364 ± 0.017</td>
<td>2.82 ± 0.092b (−9%)</td>
<td>0.013 ± 0.001b</td>
<td>0.108 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>(-7%)</td>
<td></td>
<td>(-9%)</td>
<td>(+7%)</td>
<td>(+32%)</td>
<td>(-38%)</td>
<td>(-7%)</td>
</tr>
</tbody>
</table>

CP: cisplatin treated; CPFXO: flaxseed oil + cisplatin treated; FXO: flaxseed oil diet; LDH: lactate dehydrogenase; MDH: malate dehydrogenase; HK: hexokinase; G6Pase: glucose-6-phosphatase; FBPase: fructose-1,6-bisphosphatase; G6PDH: glucose-6-phosphatase dehydrogenase; ME: malic enzyme.

*Results are mean ± SEM for five different experiments. Values in parentheses represent percent change from control.

bSignificantly different from control at p < 0.05 by one-way ANOVA.

Significantly different from CP at p < 0.05 by one-way ANOVA.
**Table 5. Effect of flaxseed oil (FXO) on enzymatic and nonenzymatic antioxidant parameters with/without CP treatment in liver homogenates**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Lipid peroxidation (nmol/g tissue)</th>
<th>Total SH (μmol/g tissue)</th>
<th>SOD (μmol/mg protein)</th>
<th>Catalase (μmol/mg protein/ min)</th>
<th>GSH-Px (μmol/mg protein/ min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>96.22 ± 3.92</td>
<td>15.26 ± 0.38</td>
<td>74.11 ± 3.79</td>
<td>2.46 ± 0.11</td>
<td>0.116 ± 0.002</td>
</tr>
<tr>
<td>CP</td>
<td>145.89 ± 1.68&lt;sup&gt;b&lt;/sup&gt; (+52%)</td>
<td>10.66 ± 0.32&lt;sup&gt;b&lt;/sup&gt; (−30%)</td>
<td>63.17 ± 3.59&lt;sup&gt;b,c&lt;/sup&gt; (−15%)</td>
<td>0.753 ± 0.126&lt;sup&gt;b&lt;/sup&gt; (−70%)</td>
<td>0.028 ± 0.0012&lt;sup&gt;b&lt;/sup&gt; (−76%)</td>
</tr>
<tr>
<td>CPFXO</td>
<td>114.41 ± 1.34&lt;sup&gt;b,c&lt;/sup&gt; (+19%)</td>
<td>14.50 ± 0.89 (−5%)</td>
<td>69.37 ± 3.59&lt;sup&gt;b,c&lt;/sup&gt; (−6%)</td>
<td>1.85 ± 0.135&lt;sup&gt;c&lt;/sup&gt; (−25%)</td>
<td>0.086 ± 0.00057&lt;sup&gt;b,c&lt;/sup&gt; (−26%)</td>
</tr>
<tr>
<td>FXO</td>
<td>50.51 ± 2.52&lt;sup&gt;b&lt;/sup&gt; (−47%)</td>
<td>17.96 ± 0.317&lt;sup&gt;b&lt;/sup&gt; (+18%)</td>
<td>83.67 ± 1.54&lt;sup&gt;b&lt;/sup&gt; (+13%)</td>
<td>3.63 ± 0.07&lt;sup&gt;b&lt;/sup&gt; (+48%)</td>
<td>0.138 ± 0.0029&lt;sup&gt;b&lt;/sup&gt; (+19%)</td>
</tr>
</tbody>
</table>

CP: cisplatin treated; CPFXO: flaxseed oil + cisplatin treated; FXO: flaxseed oil diet; GSH-Px: glutathione peroxidase; LPO: lipid peroxidation; SOD: superoxide dismutase; total −SH: sulphydryl groups.

<sup>a</sup>Results are mean ± SEM for five different experiments. Values in parentheses represent percent change from control.

<sup>b</sup>Significantly different from control at p < 0.05 by one-way ANOVA.

<sup>c</sup>Significantly different from CP at p < 0.05 by one-way ANOVA.
PUFA are plants/seed namely flaxseed and fish/marine foods. ω-3 PUFA-enriched FO has received a great deal of attention as therapeutic options in a variety of clinical situations. Lately, flaxseed (L. usitatissimum) has been the focus of increased interest in the field of diet and disease research due to the potential health benefits associated with its biologically active component viz ω-3 PUFA. FXO has been shown to prevent lipid disorders and lead-induced neurotoxicity and nephrotoxicity. However, the role of FXO in preventing drug-induced toxicity has not yet been investigated.

The present work was undertaken to study detailed mechanism of CP-induced hepatotoxic alterations and possible role of FXO in preventing those deleterious changes in rat liver. Single CP injection caused marked alterations in serum and urine parameters. The present results show that CP administration to control rats caused hepatotoxicity as indicated by elevated levels of transaminases (ALT and AST). Moreover, Scr and BUN were also enhanced with significant decrease in creatinine clearance. These results also show a significant decrease in serum glucose, Pi, PLs and serum cholesterol, accompanied by massive proteinuria, glucosuria and phosphaturia. FXO diet given prior to and following CP administration prevented CP-induced alterations in various serum/urine parameters. CP elicited increase in levels of Scr, BUN and transaminases were lowered when CP was administered to FXO fed rats. Serum glucose, PLs and phosphate were improved upon CP treatment to FXO fed rats.

Since the brush border membrane of renal proximal tubules has been shown as the major site of CP-induced renal injury, the integrity of the membrane was assessed by the status of its biomarker enzymes ALP, GGTase and LAP. CP significantly decreased the activities of ALP, GGTase and LAP in the liver homogenate. The decrease in membrane enzyme activities might have occurred due to the loss of membrane enzyme and other proteinic components, indicating adverse effects of CP on membrane integrity. The present results show that in contrast to CP treatment, dietary supplementation of FXO in control rats caused significant increase in the activities of membrane enzymes in the liver homogenate. Dietary FXO supplementation prior to and following CP treatment prevented/retarded CP-induced decrease in membrane enzymes in the tissue. The activity of lysosomal enzyme ACPase was significantly increased in liver homogenate by CP treatment. Alteration in ACPase activity demonstrate CP-induced loss of lysosomal function. However, the CP-induced effect on lysosomal enzyme activity appeared to be ameliorated by dietary FXO supplementation.

To assess the functional aspects, the activities of various metabolic enzymes were determined under different experimental conditions. The activities of various enzymes involved in glycolysis, tricarboxylic acid (TCA) cycle, gluconeogenesis and hexose monophosphate (HMP)-shunt pathways were differentially altered by CP treatment and/or by FXO consumption. CP caused significant increase in LDH and decrease in MDH which was accompanied with a simultaneous increase in HK activity in liver tissue. Although the actual rates of glycolysis or TCA cycle were not determined, marked decrease in MDH activity appears to be due to CP-induced damage to mitochondria.

A marked increase in LDH activity with simultaneous decline in TCA cycle enzyme, MDH appears to be an adaptive cellular effect in energy metabolism from aerobic metabolism alternatively to anaerobic glycolysis due to CP-induced mitochondrial dysfunction.

CP also altered the activities of enzymes of gluconeogenesis and HMP-shunt pathway. The activities of G6Pase, FBPase and G6PDH were profoundly decreased albeit to different extent. However, the activity of NADP ME was increased in the tissue. The present data indicate that CP caused differential effects on different enzymes of carbohydrate metabolism. FXO administration to CP-treated rats resulted in the overall improvement of carbohydrate metabolism as evident by higher activities of MDH and gluconeogenic enzymes in CPFXO as compared to CP group. FXO might have lowered the number of damaged mitochondria or other affected macromolecules or may have increased the number of normally active organelles or macromolecules.

Earlier reports reveal that heavy metals including CP exert their toxic effects by inducing the generation of ROS. A major cellular defense against ROS is provided by SOD and CAT, which together convert superoxide radicals first to H2O2 and then to molecular oxygen and water. Other enzymes, e.g. GSH-Px, use thiol-reducing power of glutathione to reduce oxidized lipids and protein targets of ROS. However, oxidative stress can occur as a result of either increased ROS generation and/or decrease in antioxidant enzyme system. These antioxidant enzymes protect the cell against cytotoxic ROS. In agreement with the previous studies present results show that CP enhanced LPO, an indicator of tissue injury and depleted protein thioles. CP administration to control rats caused severe
damage to liver tissue most likely by ROS generation as apparent by perturbation in the antioxidant enzymes (SOD, CAT and GPx-SH) and total-SH content that lead to increased LPO. CP-treated rats fed on FXO-supplemented diet caused significant increase in SOD, CAT and GSH-Px activities accompanied by lower LPO values in liver tissue. The protection against CP by FXO can be attributed to its intrinsic biochemical and natural antioxidant properties. It appears that FXO enriched with ω-3 fatty acids enhanced resistance to free radical attack generated by CP administration. As indicated in Figure 3, our results indicate an overall improvement in metabolic activities, lysosomal integrity and augmentation of antioxidant defenses in CP-treated FXO-fed rats.

We conclude that while CP elicited deleterious hepatotoxic effects by causing severe damage to the plasma membrane, mitochondria and other organelles by suppressing antioxidant defense mechanism, however these effects were ameliorated by dietary supplementation with FXO. Present study thus support the rationale that ω-3 fatty acid–enriched FXO may be effective dietary supplementation to maximize the clinical use of CP in the treatment of various malignancies without hepatotoxic and other side effects.

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**Declaration of Conflict of Interest**

The authors declared no conflicts of interest.

**References**


